DECLARATION

I, the below-named translator, hereby declare:

(1) That my name, mailing address and citizenship are as stated below;

(2) That I am knowledgeable in the English language and in the Korean language in which Korean Patent Application No. 10-2002-0041764 was filed on July 16, 2002; and

(3) That I have translated said Korean Patent Application No. 10-2002-0041764 into English, which English text is attached hereto, and believe that said translation is a true and complete translation of the aforementioned Korean patent application.

June 1, 2007

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KOREAN INDUSTRIAL PROPERTY OFFICE

This is to certify that the following application annexed hereto is a true copy from the records of the Korean Industrial Property Office.

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Applicant(s): PanGenomics Co., Ltd.

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FILING DETAILS OF THE PATENT APPLICATION

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[TITLE OF THE INVENTION]
Her-2/neu DNA VACCINE HAVING ANTI-CANCER EFFECT
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[NUCLEIC ACID SEQUENCE LISTINGS OR AMINO ACID SEQUENCE LISTINGS]

[NUMBER OF SEQUENCE] 9
[SEQUENCE LISTINGS ELECTRONIC FILE] ATTACHED

The above application is filed in accordance with Article 42 of Korean Patent Law.

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ABSTRACT

The present invention relates to a plasmid having anti-cancer effect, which comprises human Her-2/neu gene lacking the intracellular domain, and a composition and DNA vaccine comprising same for preventing or treating cancer. The Her-2/neu DNA vaccines of the present invention having excellent anti-cancer effect can be effectively used as a therapeutic vaccine in reducing metastasis after tumor surgery or as a prophylactic vaccine for people with genetic high risk.

Representative Figure

Fig. 1a

SPECIFICATION

Title of the Invention

Her-2/neu DNA VACCINE HAVING ANTI-CANCER EFFECT

Brief Description of the Drawings

Figs. 1a and 1b: a schematic procedure for preparing pNeu plasmid construct (A) and a preset immunization schedule (B), respectively;

Figs. 2a to 2e: a graph showing antibody response induced by pTV2, $pNeu_{TM}$, $pNeu_{ECD}$, $pNeu_{TM\text{-}gDs}$ and $pNeu_{ECD\text{-}gDs}$, respectively (white histogram: control antibody; and black histogram: diluted test sera);

Figs. 3a to 3e: a graph comparing Her-2/neu-specific antibody responses induced by each plasmid (A: PBS; B: $pNeu_{ECD}$; C: $pNeu_{TM}$; D: pCK_{ECD}; E: pCK_{TM}; white histogram: control antibody; and black histogram: test sera);

Figs. 4a to 4c: confocal microscopic analysis of mouse sera immunized with pTV2, $pNeu_{TM}$ and $pNeu_{ECD\text{-}gDs}$, respectively;

Figs. 5a to 5e: a graph showing cytotoxic T lymphocytes (CTL) responses induced by pTV2, $pNeu_{TM}$, $pNeu_{ECD}$, $pNeu_{TM\text{-}gDs}$ and $pNeu_{ECD\text{-}gDs}$, respectively;

Figs. 6a to 6e: a graph comparing CTL responses induced by each plasmids (A: PBS; B: $pNeu_{ECD}$; C: $pNeu_{TM}$; D: pCK_{ECD}; and E: pCK_{TM});

Figs. 7a and 7b: preventive anti-cancer effect induced by pNeu plasmids; tumor size in subcutaneous injection model of Her2-CT26 cells after administering each plasmids (7a), and survival rate of mouse in intravenous injection model of Her2-CT26 cells in accordance with time after administering each plasmids (7b);

Figs. 8a and 8b: preventive anti-cancer effect induced by pCK_{ECD} and pCK_{TM} plasmids; tumor size in subcutaneous injection model of Her2-CT26 cells after administering each plasmids (8a), and survival rate of mouse in intravenous injection model of Her2-CT26 cells in accordance with time after administering each plasmids (8b);

Figs. 9a and 9b: preventive anti-cancer effect induced by $pNeu_{ECD}$ and

$pNeu_{ECD-gDs}$ plasmids; tumor size in subcutaneous injection model of Her2-CT26 cells after administering each plasmids (9a), and survival rate of mouse in intravenous injection model of Her2-CT26 cells in accordance with time after administering each plasmids (9b);

Figs. 10a and 10b: therapeutic anti-cancer effect induced by $pNeu_{ECD}$ and $pNeu_{ECD-gDs}$ plasmids, wherein 10a and 10b, survival rates of mouse in intravenous injection model of 1×10^5 or 5×10^5 Her2-CT26 cells in accordance with time after administering each plasmids; and

Fig. 11: therapeutic anti-cancer effect induced by pCK_{ECD} and pCK_{TM} plasmids.

Detailed Description of the Invention

Purpose of the Invention

Field of the Invention and Prior Arts

The present invention relates to a plasmid having anti-cancer effect, which comprises human Her-2/neu DNA, and a vaccine comprising same.

The Her-2/neu encodes a transmembrane protein (185 kDa) that is a member of the type I family of growth factor receptors (Akiyama, T. et al., *Science* 232:1644-1646, 1986; and Bargmann, C. I., et al., *Nature*, 319:226-230, 1986). Amplification of this gene results in overexpression of the encoded 185 kDa tyrosine kinase, and it is similar with epidermal growth factor receptor (Coussens, L., et al., *Science*, 230:1132-1139, 1985; Stern, D. F., et al., *Mol. Cell Biol.*, 6: 1729-1740, 1986; and Kraus, M. H., et al., *Embo. J.*, 6:605-610, 1987).

The Her-2/neu protein has been found to be amplified and overexpressed in several types of human adenocarcinomas, especially in tumors of the breast and the ovary (Kraus, M. H., et al., *supra*; Slamon, D. J., et al., *Science*, 244:707-712. 1989; and Yonemura, Y., et al., *Cancer Res.*, 51:1034-1038, 1991). The overexpression of Her-2/neu was correlated with short relapse time and poor survival rate of breast cancer patients (Slamon, D. J. et al., *Science* 235:177-182, 1987; Press, M. F., et al., *Cancer Res.*, 53:4960-4970, 1993; and Seshadri, R., et al., *J. Clin. Oncol.*, 11:1936-1942, 1993), suggesting that Her-2/neu overexpression itself likely plays a critical role in the

development of human cancers. Several lines of evidence also support a direct role of Her-2/neu in the pathogenesis and clinical aggressiveness of Her-2/neu-expressing tumors (Baselga, J., et al., *Semin. Oncol.*, 26:78-83, 1999). Further, Her-2/neu-specific antibodies and T cells are detected in breast and ovarian cancer patients (Disis, M. L., et al., *Cancer Res.*, 54:16-20, 1994; Disis, M. G., et al., *J. Clin. Oncol.*, 15:3363-3367, 1997; Peoples, G. E., et al., *Proc. Natl. Acad. Sci. USA*, 92:432-436, 1995; Kono, K., et al., *Int. J. Cancer*, 70:112-119, 1997; Kobayashi, H., et al., *Cancer Res.*, 60:5228-5236, 2000; and Charo. K., et al., *J. Immunol.*, 163:5913-5919, 1999). Therefore, Her-2/neu oncogene is an excellent target for the development of therapeutic vaccines specific for Her-2/neu-overexpressing cancers.

It is well known that human Her-2/neu gene has tyrosine kinase activity in the intracellular domain and its overexpression itself stimulates abnormal cell division (Coussens, L., et al., *Science*, 230:1132-1139, 1985; Stern, D. F., et al., *Mol. Cell Biol.*, 6: 1729-1740, 1986; and Kraus, M. H., et al., *Embo. J.*, 6:605-610, 1987). Therefore, there are several attempts to inhibit possible oncogenecity caused by using entire molecule in a vaccine by introducing a mutation into the cytoplasmic kinase active domain to inhibit tyrosine kinase activity (Wei, W. I. et al., *Int. J. Cancer* 81: 748-754, 1999) or by using truncated molecules lacking the intracellular or extracellular domain.

Plasmids are relatively simple to generate and safe so that they can be attractive vectors for the development of anti-cancer vaccines encoding tumor-associated antigens. Because they are not proteins nor associated with a viral coat, they do not produce neutralizing antibodies that can hamper the clinical efficacy of vaccines (Hellstrom, I. and Hellstrom, K. E., *J. Immunother.* 21:119-126, 1998; and Minev, B. R., *Pharmacol. Ther.*, 81:121-139, 1999).

In preclinical tumor models, DNA vaccines encoding rat Her-2/neu (Chen, Y. et al., *Cancer Res.*, 58:1965-1971, 1998; Amici, A., et al., *Gene Ther.*, 7:703-706, 2000; and Rovero, S., et al., *J. Immunol.*, 165:5133-5142, 2000) or human Her-2/neu (Foy, T. M., et al., *Vaccine*, 19:2598-2606, 2001; Piechocki, M. P., et al., *J. Immunol.*, 167:3367-3374, 2001; and Pilon, S. A. et al., *J. Immunol.*, 167:3201-3206, 2001) induced preventive effect against Her-2/neu over-expressing cancer cells.

Although successful anti-cancer effects against Her-2/neu expressing cancer cells by Her-2/neu DNA vaccine have been achieved by many earlier experiments (Amici, A., et al., *supra*; Pupa, S. M., et al., *Gene Ther.*, 8:75-59, 2001; Lachman, L. B., et al., *Cancer Gene Ther.*, 8:259-268, 2001; and Wei, W. Z., et al., *Int. J. Cancer*, 81:748-754, 1999), no successful therapeutic effect has been reported. The difficulty lies on the slow gain of antitumor immunity due to the lag time before antigenic expression of Her-2/neu expressing plasmids, while breast cancer cell grows relatively fast. Therefore, some of the Her-2/neu therapeutic vaccine experiments were conducted by using cells (Valone, F. H., et al., *Cancer J.*, 7. Suppl 2:S53-61, 2001), the combination of DNA and cytokine-secreting cancer cells (Chen, S. A. et al., *Clin. Cancer Res.*, 6:4381-4388, 2000), or dendritic cell (Chen, Y., *Gene Ther.*, 8:316-323, 2001).

Therefore, the present inventors have endeavored to develop Her-2/neu DNA vaccines having high anti-cancer activity which can be effectively used as a DNA vaccine for preventing and treating cancer.

Technical Object of the Invention

Accordingly, it is an object of the present invention to provide a human Her-2/neu expressing plasmid construct having high antitumor activity.

It is another object of the present invention to provide a composition comprising the plasmid construct as an active ingredient for preventing and/or treating cancers.

It is further object of the present invention to provide a vaccine comprising the plasmid construct as an active ingredient for preventing and/or treating cancers.

Constitution of the Invention

In accordance with one aspect of the present invention, there is provided a plasmid construct having anti-cancer activity, which is prepared by inserting a truncated human Her-2/neu gene lacking the intracellular domain into pTV2 or pCK vector.

The truncated Her-2/neu gene lacking the intracellular domain has the nucleotide sequence of SEQ ID NO: 2, and is inserted into pTV2 vector (Lee, S. W. et al., *J. Virol.*, 72:8430-8436, 1998) or pCK vector (Accession No.: KCCM-10179), which has a high expression level *in vivo*, preferably, into *Kpn*I/*Xba*I site (without gDs) or *Asc*I/*Xba*I site (with gDs).

In the present invention, deleting the intracellular domain from human Her-2/neu gene of the inventive plasmid has the following advantages. Namely, possible oncogenecity of Her-2/neu can be eliminated by constructing truncated Her-2/neu plasmids lacking the Her-2/neu cytoplasmic kinase domain to block the abnormal growth signal transduction caused by the cytoplasmic kinase domain. In addition, the truncated Her-2/neu of the present invention enables to avoid the dangers of autoimmunity against the Her-2/neu intracellular domain that is highly conserved among the members of the EGFR (epidermal growth factor receptor) family. DNA vaccines using the truncated Her-2/neu lacking the intracellular domain in order to exclude the risks of oncogenecity and autoimmunity have been reported (Chen, Y. et al., *Cancer Res.*, 58:1965-1971, 1998; and Amici, A., et al., *Gene Ther.*, 7:703-706, 2000), but they did not have excellent anti-cancer effect. Whereas, the inventive plasmid has excellent anti-cancer effect by inducing both antibody response and CTL response, and shows therapeutic effect against metastatic cancer.

The present invention also provides a plasmid constructs encoding the truncated human Her-2/neu gene of SEQ ID NO: 3 that lacks the transmembrane domain from the above plasmid, which results in the secretion of the expressed protein into the cell exterior.

Further, the inventive plasmid can be prepared by replacing the inherent signal sequence of human Her-2/neu gene with exogenous signal sequence, such as the herpes simplex virus type I glycoprotein D signal (gDs) sequence which is known to facilitate the efficient expression and secretion of human immunodeficiency virus (HIV) type I gp160.

In a preferred embodiment of the present invention, $pNeu_{TM}$ and pCK_{TM} prepared by inserting the truncated Her-2/neu gene lacking the intracellular domain to pTV and pCK vectors, respectively; $pNeu_{ECD}$ and pCK_{ECD} prepared by deleting the transmembrane domain of Her-2/neu gene from $pNeu_{TM}$ and pCK_{TM},

respectively; and $pNeu_{TM-gDs}$ and $pNeu_{ECD-gDs}$ prepared by replacing the inherent Her-2/neu signal peptide sequence of $pNeu_{TM}$ and $pNeu_{ECD}$ with the signal sequence of glycoprotein D of herpes simplex virus type I.

The plasmids, $pNeu_{TM}$, pCK_{TM}, $pNeu_{ECD}$ and pCK_{ECD} have been deposited on June 26, 2002 with the Korean Culture Center of Microorganisms (KCCM) under the accession numbers KCCM-10393, KCCM-10396, KCCM-10394 and KCCM-10395 respectively.

Administration of the plasmids to BALB/c mouse induce Her-2/neu specific IgG antibody according to the signal peptide sequence in various aspects. Specifically, Her-2/neu specific IgG titer is very high in serum of the mouse administered with $pNeu_{TM}$ or $pNeu_{ECD}$, but it is relatively low in serum of the mouse administered with $pNeu_{TM-gDs}$ or $pNeu_{ECD-gDs}$. On the other hand, all plasmids induce strong Her-2/neu-specific CTL response. Accordingly, a relative importance of Her-2/neu specific CTL and antibody was evaluated by using these plasmids to eliminate Her-2/neu-expressing cancer cell. As a result, these plasmids induce complete protection against a small number of cancer cells, and anti-cancer effect of $pNeu_{ECD}$ and $pNeu_{ECD-gDs}$ are not significantly different in a preventive and therapeutic model. However, when a large number of cancer cells are used in a therapeutic model, only $pNeu_{ECD}$ shows statistically significant anti-cancer effect. This supports that only strong CTL response is enough to prevent cancer, but both CTL and antibody are necessary to treat cancer.

Meanwhile, plasmids pCK_{TM} and pCK_{ECD} show almost similar effect to those of $pNeu_{TM}$ and $pNeu_{ECD}$, and this suggests that the inventive plasmid can be clinically used as a vaccine for treating cancer.

The composition comprising the plasmid can be used as a therapeutic vaccine in reducing metastasis after tumor surgery and as a prophylactic vaccine for people with genetic high risk.

The composition of the present invention may be formulated for oral or parenteral administration. The formulation for oral administration may take various forms such as tablets, peels, soft and hard capsules, aqueous solutions, suspensions, emulsions, syrups and granules, which may contain conventional additives such as a diluent (e.g., lactose, dextrose, sucrose, mannitol, sorbitol,

cellulose and/or glycine), a lubricant (e.g., silica, talc, stearic acid and its magnesium or calcium salts, and/or polyethylene glycol). In the case of the tablet form, the composition may further comprise a binder (e.g., magnesium aluminum silicate, starch paste, gelatin, tragacanth, methyl cellulose, sodium carboxymethyl cellulose and/or polyvinyl pyrrolidone) and optionally a disintegrant (e.g., starch, agar and alginic acid or its sodium salt), absorbent, colorant, flavor, sweetener and the like. The formulation may be prepared by conventional methods for mixture, granulation or coating. Further, the formulation for parenteral administration may take injection formulation such as isotonic aqueous solution or suspension.

The composition may be sterilized and/or contain an adjuvant such as a preservative, stabilizer, wetting agent, emulsifier, a salt for controlling an osmotic pressure and/or a buffer solution, and other therapeutically effective materials, and prepared in accordance with the conventional methods.

The inventive compounds as an active ingredient may be administered through oral route or parenteral route in an effective amount ranging from about 0.2 to 10 mg/kg (body weight), preferably from about 4 to 5 mg/kg (body weight) per day in a single dose or in divided doses in case of a mammal including a human being.

The following Examples are intended to further illustrate the present invention without limiting its scope.

<u>Reference Example 1</u>: Cell lines and animals

The Her-2/neu expressing human breast carcinoma SK-BR3 cell line and murine colon adenocarcinoma cell line CT26 were obtained from the American Type Culture Collection (Manassas, VA, USA) (ATCC HTB-30 and ATCC CRL-2639). Human breast cancer cell line SK-BR3 cells were maintained in RPMI1640 (BioWhittaker, Walkersvile, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Gaithersburg, MD) and 1% penicillin-streptomycin (GIBCO). Her-2/neu-expressing transfectoma Her-2/CT26 cells were prepared by transduction of CT26 cells with the cDNA-

encoding human Her-2/neu (NCBI: M11730). Her-2/CT26 and CT26 cells were cultured in IMDM (BioWhittaker) containing 10% heat-inactivated FBS (GIBCO, Gaithersburg, MD) and 1% penicillin-streptomycin (GIBCO).

Female 5-week-old BALB/C mice were purchased from Charles River (Osaka, Japan) and kept at 22℃, 55% relative humidity, and a daily lighting cycle of 12hrs light/ 12hrs dark with free access to food and water. The mice were housed at Laboratory Animal Center of Seoul National University until use and kept in a germ-free isolator during the whole experiments.

Reference Example 2: Isolation of DNA plasmids for i.m. injection

Escherichia coli strain DH5 transformed with each of the plasmids, $pNeu_{TM}$, $pNeu_{ECD}$, $pNeu_{TM\text{-}gDs}$, $pNeu_{ECD\text{-}gDs}$, pCK_{TM}, pCK_{ECD}, and control vectors pTV2 and pCK, was grown in LB broth (Difco, Detroit, MI). Large-scale preparation of the plasmid DNA was carried out by the alkaline lysis method using an Endofree Qiagen Plasmid-Giga kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. DNA was then precipitated, suspended in sterile PBS (BioWhittaker) at a concentration of 2 ㎎/㎖, and stored in aliquots at –20℃ for subsequent use in immunization protocols.

Reference Example 3: Flow cytometry (FACS)

To examine whether antibody in serum could specifically reacts with Her-2/neu surface protein, SK-BR3, Her-2/CT26 and CT26 cells were stripped from the culture flasks with a cell scraper (Nunc, Naperville, IL). Removed cells were washed in a buffer consisting of RPMI1640, 2% FBS and 0.1% sodium azide. Approximately 2×10^5 cells per analysis were incubated together with a serial dilute of an antibody or control antibody at 4℃ for 30 min. Cells were washed with the buffer 3 times and then stained with an FITC-conjugated goat monoclonal antibody specific for mouse IgG (Sigma) at 4℃ for 30 minutes. The stained cells were washed with the buffer 2 times and resuspended with the buffer. To exclude dead cells from data, 1 ㎍/㎖ propidium iodide (Sigma) was added to the cell suspension and incubated for 5

minutes prior to analysis. Only the cells that were negative by propidium iodide staining were gated and further analyzed for binding to cancer cells. Flow cytometry was performed using a PAS IIIi flow cytometer (Partec GmbH, Münster, Germany).

Reference Example 4: Confocal microscopy for anti-Her-2/neu antibodies

Approximately 1×10^5 SK-BR3 cells were grown for three days on Lab-Tek chambered coverglass (Nunc, Naperville, IL) coated with 1 ㎎/㎖ poly-L-Lysine. The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, washed three times with DMEM (BioWhittaker, Walkersvile, MD), blocked with DMEM containing 1% goat-globulin at 4℃ for 1 hour, incubated with 1:50 diluted mouse sera in a blocking solution at 4℃ for 8 hours, washed, and incubated with R-phycoerythrin-conjugated goat anti-mouse immunoglobulin secondary antibody (Southern Biotech, Birmingham, AL) at room temperature for 30 min. Slides were then mounted on Gel/Mount media (Fisher) and examined using a confocal microscopy (Leica TCS-SP laser scanning microscopy).

Reference Example 5: DNA immunization method

100 ㎍ of plasmid DNA that was dissolved in 100 ㎕ of sterile PBS was injected intramuscularly to two anterior tibialis of each mouse. The injection site was anesthetized with bupivacaine-HCl (ASTRA, Westborough, MA). For daily immunization for therapeutic vaccination, bupivacaine-HCl was pretreated only once just before the first immunization. Sera were collected at selected time points and monitored for the presence of anti-Her-2/neu antibodies.

Reference Example 6: Chromium-release assays

Splenocytes prepared by extracting spleen from immunized mice were cultured with mytomycin C (Sigma) treated Her-2/CT26 cells for 6 days. Her-

2/CT26 or CT26 tumor target cells were labeled with ^{51}Cr by incubating 2×10^6 cells with 200 μCi $Na^{51}CrO_4$ in 200 ㎕ saline at 37℃ for 90 min. The unincorporated ^{51}Cr was removed by washing with RPMI1640 four times. After 6 days, the serially diluted splenocytes were mixed with 10,000 labeled target cells in RPMI supplemented with 10% FBS in the wells of a round-bottom microtiter plate. The plate was incubated at 37℃ for 4 hours. After the incubation, the plate was centrifuged, and a 100 ㎕ aliquot was removed from each well for counting with a scintillation counter (Packard, Minaxi Auto Gamma 5000 Series). The percent lysis was calculated by formula 1:

<Formula 1>

Specific lysis (%) = $100 \times [(cpm_{experimental} - cpm_{spontaneous}) / (cpm_{max} - cpm_{spontaneous})]$

The cpm_{max} value was determined by adding 10 ㎕ of 5% triton-X (Sigma) to the wells containing ^{51}Cr-labeled target cells. Each group was tested in duplicate. The $cpm_{spontaneous}$ value was determined by adding only an equal volume of the medium without the addition of splenocytes or triton-X.

Reference Example 7: Tumor challenge

Mice were challenged by injection with Her-2/CT26 cells suspended in sterile PBS either subcutaneously on the flank or intravenously. The three-dimensional size of each tumor was measured with a caliper, and the volume was calculated by formula 2:

<Formula 2>

tumor volume (mm^3) = (width $\times$ length $\times$ depth) $m^3 \times (1/2)^3 \times 4\pi/3$

Animals were palpated twice a week for the development of tumors. Mice showing any symptom of acute sickness, hard to breathe or rare movement were sacrificed.

Example 1: Construction of Her-2/neu expressing plasmids

The cDNA encoding the entire human Her-2/neu gene (SEQ ID NO: 1) was inserted into *Hin*dIII/*Xba*I site of the pRC/CMV (Invitrogen Life technologies) to produce a full-length Her-2/neu plasmid (9.6 Kb).

The plasmid $pNeu_{ECD}$ and pCK_{ECD} comprising the extracellular domain of Her-2/neu without the intracellular and transmembrane domains of Her-2/neu, was generated from the PCR product of the obtained full-length Her-2/neu plasmid (9.6 Kb) using NF6 (SEQ ID NO: 4) and NSR1 (SEQ ID NO: 5) as a primer pair, and cloned into the *Kpn*I and *Xba*I sites of pTV2 (Lee, S. W., et al., *J. Virol.*, 72:8430-8436, 1998) and pCK (Lee, Y., et al., *Biochem. Biophys. Res. Commun.*, 272:230-235, 2000; Accession No.: KCCM-10179), respectively. PCR was carried out at 94℃ for 2 min; 25 cycles of 94℃ for 15 sec, 55℃ for 30 sec and 68℃ for 3.5 min; and 72℃ for 7 min.

Further, in order to obtain plasmid $pNeu_{TM}$ and pCK_{TM} encoding the extracellular and transmembrane domains of Her-2/neu without the intracellular domain of Her-2/neu, the PCR was repeated except for using NF5 SEQ ID NO: 6) and NRM2 (SEQ ID NO: 7) as a primer pair, and the PCR product was cloned into the *Kpn*I and *Xba*I sites of pTV2 and pCK, respectively (Fig. 1a).

Then, in order to obtain the plasmid $pNeu_{ECD\text{-}gDs}$, encoding the extracellular domain of Her-2/neu without the intracellular and transmembrane domains of Her-2/neu and the herpes simplex virus type I glycoprotein D (gD) signal sequence instead of Her-2/neu signal sequence, PCR was repeated except for using NSF2 (SEQ ID NO: 8) and NSR1 (SEQ ID NO: 5) as a primer pair, and the PCR product was cloned into the *Kpn*I and *Xba*I sites of pTV2-gDs (herpes simplex virus type I glycoprotein D (gD) signal sequence was cloned into pTV2 vector). Further, in order to obtain the plasmid $pNeu_{TM\text{-}gDs}$, encoding the extracellular and transmembrane domains of Her-2/neu and the gD signal sequence instead of Her-2/neu signal sequence, PCR was repeated except for using NF3 (SEQ ID NO: 9) and NRM2 (SEQ ID NO: 7) as a primer pair, and cloned into the *Asc*I and *Xba*I sites of pTV2-gDs (Fig. 1a).

Example 2: Induction of Her-2/neu specific antibody by Her-2/neu DNA

vaccination

Whether various pNeu plasmid constructs could induce Her-2/neu specific antibodies were examined as follows. 100 μg of plasmid DNA was injected intramuscularly to each mouse prepared in Reference Example 1 three times according to a preset immunization schedule (Fig. 1b). Some mice of each group were sacrificed to obtain spleen, and Her-2/neu-specific CTL was determined. Other mice were challenged with Her-2/neu expressing cancer cell to evaluate anti-cancer immunity.

Sera were obtained from mice before the first injection and 7 days after the third injection. The Her-2/neu specific antibody titer in the serum was measured based on the binding of the antibody to the breast cancer cell line, SK-BR3, using a flow cytometry as described in Reference Example 3. Her-2/neu specific antibody titers of all mice vaccinated with $pNeu_{TM}$, $pNeu_{TM\text{-}gDs}$, $pNeu_{ECD}$ or $pNeu_{ECD\text{-}gDs}$ were determined, and the greatest dilution of serum for which a shift in the mean fluorescence intensity by the binding to SK-BR3 cells, compared to that of control antibody, was shown in Table 1.

<Table 1>

pTV2 (n=5)	$pNeu_{TM}$ (n=5)	$pNeu_{ECD}$ (n=5)	$pNeu_{TM\text{-}gDs}$ (n=5)	$pNeu_{ECD\text{-}gDs}$ (n=5)
<50	12800	12800	800	<50
<50	12800	12800	50	<50
<50	3200	12800	<50	<50
<50	12800	12800	800	<50
<50	3200	12800	50	<50

As shown in Table 1, the observed Her-2/neu specific antibody titers were ranked in the order of $pNeu_{ECD}$ > $pNeu_{TM}$ > $pNeu_{TM\text{-}gDs}$ > $pNeu_{ECD\text{-}gDs}$ $\gg$ pTV2. As expected, none of the sera collected from animals before the injection of plasmid DNA had detectable Her-2/neu specific binding activities. Moreover, none of the animals injected with pTV2 made detectable anti-Her-2/neu antibodies at 1:50 dilution (Fig. 2a). However, vaccination with $pNeu_{TM}$ or $pNeu_{ECD}$ resulted in high Her-2/neu specific IgG titers, and serum samples

diluted by 1:800 revealed a wide shift in the mean fluorescence intensity (Figs. 2b and 2c). In contrast, vaccination with $pNeu_{TM-gDs}$ or $pNeu_{ECD-gDs}$ resulted in a very low IgG titer, and serum samples diluted by 1:50 revealed a little or a barely detectable shift in the mean fluorescence intensity (Figs. 2d and 2e). Serum samples obtained from immunized mice 10 days after the third injection with plasmid pCK_{TM} and pCK_{ECD}, respectively and diluted by 1:400 revealed a wide shift in the mean fluorescence intensity. Therefore, it is confirmed that the plasmids induce Her-2/neu specific antibody (Fig. 3).

Further, the existence of Her-2/neu-specific antibodies in mouse sera immunized with $pNeu_{TM}$ or $pNeu_{ECD-gDs}$ was also confirmed by confocal microscopic analysis as described in Reference Example 4. Mouse serum immunized with $pNeu_{TM}$ (Fig. 4b) demonstrated clear localization of Her-2/neu specific antibodies bound on the surface of SK-BR3, compared with those of control vector pTV2 (Fig. 4a) or $pNeu_{ECD-gDs}$ (Fig. 4c), which is consistent with the Her-2/neu specific antibody titers presented in Fig. 2.

<u>Example 3</u>: Induction of Her-2/neu specific CTL by Her-2/neu DNA vaccination

Since Her-2/neu-specific antibody responses in vaccinated mice were fluctuated with type of pNeu constructs used in immunization as described in Example 2, Her-2/neu-specific CTL responses induced in the same mice were evaluated as follows. Splenocytes were obtained 2 weeks after the third immunization from the same mice that were tested for Her-2/neu specific antibody titers in sera. Splenocytes were cultured with mytomycin-C-treated human Her-2/neu expressing syngeneic murine transfectoma, Her-2/CT26 cells for 6 days, and were assayed for the lysis of CT26 or Her-2/CT26 target cells by chromium release assay for 4 hours. As a result, splenocytes from mice vaccinated with $pNeu_{TM}$ (Fig. 5b), $pNeu_{ECD}$ (Fig. 5c), $pNeu_{TM-gDs}$ (Fig. 5d) or $pNeu_{ECD-gDs}$ (Fig. 5e) exhibited CTL-dependent lysis of Her-2/CT26, compared with splenocytes from control vector pTV2 vaccinated control mice (Fig. 5a), and the relative strength of Her-2/neu specific CTL response was in order of $pNeu_{TM} > pNeu_{ECD} > pNeu_{TM-gDs} > pNeu_{ECD-gDs} \gg$ pTV2. Her-2/neu specific

lysis by splenocytes from mice immunized with any one of pNeu constructs was comparable to the others of pNeu constructs and were 80~90% at an E:T (effector:target) ratio of 50:1 and 60~70% at an E:T ratio of 10:1 (Figs 5b to 5e). However, splenocytes from any group of mice did not induce CTL-dependent lysis of CT26 cells.

Her-2/neu specific CTL response was assayed in case of pCK_{ECD} and pCK_{TM} prepared by replacing $pNeu_{ECD}$ and $pNeu_{TM}$ to pCK (Fig. 6). As shown in Fig. 6, pCK_{ECD} and pCK_{TM} plasmids induced CTL responses comparable to those of $pNeu_{ECD}$ and $pNeu_{TM}$, and the plasmid comprising the extracellular domain (ECD) induced higher CTL responses than those of the plasmid comprising the transmembrane domain.

In brief, all Her-2/neu expressing plasmids induced strong Her-2/neu specific CTL response, which was irrelevant to their signal peptide sequences and vectors. However, they induced significantly different Her-2/neu specific antibody responses according to their signal peptide sequences, and this result was confirmed by repeated tests.

Example 4: Prevention of tumor growth by Her-2/neu DNA vaccine

Antitumor immunity against Her-2/neu expressing syngeneic murine tumor cell line Her2-CT26 in BALB/c mouse was evaluated as follows. Initially, titration studies were performed to determine the optimal number of tumor cells to be injected s.c. or i.v. into mice to generate subcutaneous tumor formation or lung metastasis. As a result, Her-2/CT26 cells induced subcutaneous or lung metastatic tumor when 5×10^4 cells or more were injected s.c. or i.v.. Since a long survival period may help to distinguish antitumor efficacy of Her-2/neu DNA plasmids, 5×10^4 cells were chosen as the initial cell number for i.v. or s.c. tumor challenge.

Each mouse received three i.m. injections of 100 μg plasmid DNA according to a preset immunization schedule (Fig. 1b) and 10 days after the third injection of plasmid DNA, each mouse was challenged i.v. or s.c. with 5×10^4 Her-2/CT26 cells. In the above subcutaneous tumor model study, all of the animals injected with control vector, pTV2, developed palpable tumors (Fig.

7a). On the other hand, tumors were completely suppressed in all groups of mice each injected with $pNeu_{TM}$, $pNeu_{TM\text{-}gDs}$, $pNeu_{ECD}$ or $pNeu_{ECD\text{-}gDs}$ for 60 days following s.c. tumor injection. In a metastasis model, all group of mice injected with $pNeu_{TM}$, $pNeu_{TM\text{-}gDs}$, $pNeu_{ECD}$ or $pNeu_{ECD\text{-}gDs}$ survived i.v. tumor challenge (Fig. 7b). However, four of the seven mice (57%) injected with only pTV2 and all mice injected with only PBS did not survive lung metastasis.

Further, to determine the antitumor effect in case of pCK_{ECD} and pCK_{TM} prepared by replacing $pNeu_{ECD}$ and $pNeu_{TM}$ to pCK, mice were vaccinated intramuscularly three times with 100 μg plasmid DNA. The mice were challenged s.c. or i.v. with 1×10^6 Her-2/CT26 cells 2 weeks after final vaccination. In case of the s.c. injection with Her-2/CT26, solid tumors were grew all mice both injected with PBS and control pCK vector. However, five of the eight mice (62.5%) and seven of the eight mice (87.5%) injected with pCK_{ECD} and pCK_{TM} showed no tumors suggesting that the plasmids significantly inhibited tumor growth (Fig. 8a). In case of i.v. injection, all mice both injected with PBS and control pCK vector died of tumor metastasis within 17 days, but the survival rate of the mice both injected with pCK_{ECD} and pCK_{TM} was significantly increased (Fig. 8b). Accordingly, it is confirmed that the plasmid having Her-2/neu gene in the pCK vector has excellent antitumor effect as well as pNeu plasmid having Her-2/neu gene in the pTV2 vector.

<u>Example 5</u>: Comparison of antitumor immunity by $pNeu_{ECD}$ and $pNeu_{ECD\text{-}gDs}$

The number of tumor cells to be injected was increased by a factor of 100 (5×10^6) for s.c. tumor challenge and by a factor of 40 (2×10^6) for i.v. tumor challenge. It was impossible to use a cell number of more than 2×10^6 for i.v. tumor challenge because there was the danger of blood vessel blockage by excessive tumor cells injected i.v.. For comparison, a set of $pNeu_{ECD}$ and $pNeu_{ECD\text{-}gDs}$ that generated the largest difference in Her-2/neu-specific antibody titers among the four different Her-2/neu-expressing plasmids was selected.

Each mouse received three i.m. injections of 100 μg plasmid DNA

according to the same immunization schedule (Fig. 1b), and 10 days after the third injection of plasmid DNA, each mouse was challenged s.c. with 5×10^6 or i.v. with 2×10^6 Her-2/CT26. In the subcutaneous model, all eight animals injected with pTV2 developed tumors and the mean tumor volume reached over 2000 mm^3 before day 19 post s.c. tumor challenge. The mean tumor volume of eight mice injected with $pNeu_{ECD}$ was 82.2 mm^3 at day 23 and that of eight mice injected with $pNeu_{ECD\text{-}gDs}$ was 67.9 cm^3. While there was significant tumor growth suppression in mice injected with $pNeu_{ECD}$ ($p = 2.9900e\text{-}8$, Student's *t* test) or $pNeu_{ECD\text{-}gDs}$ ($p= 2.8400e\text{-}8$, Student's *t* test), the difference in the mean tumor volume between the two immunized groups was not statistical significant ($P =0.8684$, Student's *t* test). In the metastasis model, lung metastasis was inhibited until day 40 in eight of the eight mice (100%) injected with $pNeu_{ECD}$ and in seven of the eight mice (88%) injected with $pNeu_{ECD\text{-}gDs}$. All mice injected with pTV2 did not survived lung metastasis. In other words, although the survival was significantly prolonged by treatment with $pNeu_{ECD}$ ($p < 0.0001$, Mantel-Haenszel test) or $pNeu_{ECD\text{-}gDs}$ ($p < 0.0001$, Mantel-Haenszel test) compared with pTV2, there was no significant difference between $pNeu_{ECD}$ and $pNeu_{ECD\text{-}gDs}$ ($p = 0.3173$, Mantel-Haenszel test) (Figs. 9a and 9b).

Example 6: Efficacy of Her-2/neu DNA vaccine in a therapeutic model

To compare the antitumor immunity efficacies of $pNeu_{ECD}$ and $pNeu_{ECD\text{-}gDs}$ in a therapeutic model, mice were challenged with tumor cells first, and then injected with DNA plasmids. 6-week old naive mice were challenged i.v. with 1×10^5 or 5×10^5 Her-2/CT26 cells, and then were divided into 4 groups. 1 hour after the tumor injection, each mouse received the first i.m. injection of 100 μg of $pNeu_{ECD}$ or $pNeu_{ECD\text{-}gDs}$, followed by four more daily i.m injections with the same DNA plasmid.

When 1×10^5 tumor cells were injected, all mice treated with $pNeu_{ECD}$ or $pNeu_{ECD\text{-}gDs}$ survived lung metastasis for the following 40 days (Fig. 10a). However, five of the eight mice (63%) injected with only pTV2 and eight of the eight mice (100%) injected with only PBS did not survive lung metastasis.

Although $pNeu_{ECD}$ and $pNeu_{ECD\text{-}gDs}$ improved the survival rate significantly ($p=0.0085$, Mantel-Haenszel test) as compared with pTV2, there was no significant difference between $pNeu_{ECD}$ and $pNeu_{ECD\text{-}gDs}$.

On the other hand, when the number of tumor cells was increased 5 times (5×10^5 cells), only the mice injected with $pNeu_{ECD}$ exhibited an increased survival rate which was statistically significantly ($p = 0.0237$, Mantel-Haenszel test, Fig. 10b) compared with mice injected with pTV2. However, the mice injected with $pNeu_{ECD\text{-}gDs}$ did not show significantly enhanced survival ($p = 0.4628$, Mantel-Haenszel test) as compared with the mice injected with pTV2. Nonetheless, consistently with the preventive model, there was no significant difference in antitumor immunity between $pNeu_{ECD}$ and $pNeu_{ECD\text{-}gDs}$ ($p = 0.4263$, Mantel-Haenszel test).

Further, to determine the therapeutic effects of pCK_{ECD} and pCK_{TM} prepared by replacing $pNeu_{ECD}$ and $pNeu_{TM}$ to pCK, mice were challenged with i.v. with 2×10^5 Her-2/CT26 cells, and then injected with DNA plasmids. In the groups injected with pCK_{TM} and pCK_{ECD} plasmids, all mice showed significantly prolonged survival rate, while in the groups injected with PBS and pCK, all mice died of lung metastasis of cancer cells within 21 days (Fig. 11). Accordingly, it is confirmed that the plasmid having Her-2/neu gene in the pCK vector has excellent antitumor therapeutic effect as well as pNeu plasmid having Her-2/neu gene in the pTV2 vector.

Effect of the Invention

The Her-2/neu DNA vaccine of the present invention can be effectively used as a therapeutic vaccine in reducing metastasis after tumor surgery or as a prophylactic vaccine for people with genetic high risk.

What is claimed is:

1. A plasmid construct having anti-cancer activity which is prepared by inserting a truncated human Her-2/neu gene lacking the intracellular domain, which has the nucleotide sequence of SEQ ID NO: 2, into plasmid pTV2 or pCK.

2. The plasmid construct of claim 1, wherein the truncated human Her-2/neu gene further lacks the transmembrane domain, and has the nucleotide of SEQ ID NO: 3.

3. The plasmid construct of claim 1, wherein the signal peptide of the human Her-2/neu gene is replaced by the signal peptide sequence of herpes simplex type I glycoprotein D (gD).

4. The plasmid construct of any of claims 1 to 3, which is selected from the group consisting of $pNeu_{TM}$ (KCCM-10393), $pNeu_{ECD}$ (KCCM-10394), pCK_{TM} (KCCM-10396), pCK_{ECD} (KCCM-10395), $pNeu_{TM\text{-}gDs}$ and $pNeu_{ECD\text{-}gDs}$.

5. A composition for preventing and/or treating cancer, which comprises the plasmid construct of claim 1 as an active ingredient.

6. The composition of claim 5, which is an injection formulation prepared by suspending the plasmid construct of claim 1 in injectable medium.

7. A DNA vaccine for preventing and/or treating cancer, which comprises the plasmid construct of claim 1 as an active ingredient.

FIGURE

Fig. 1a

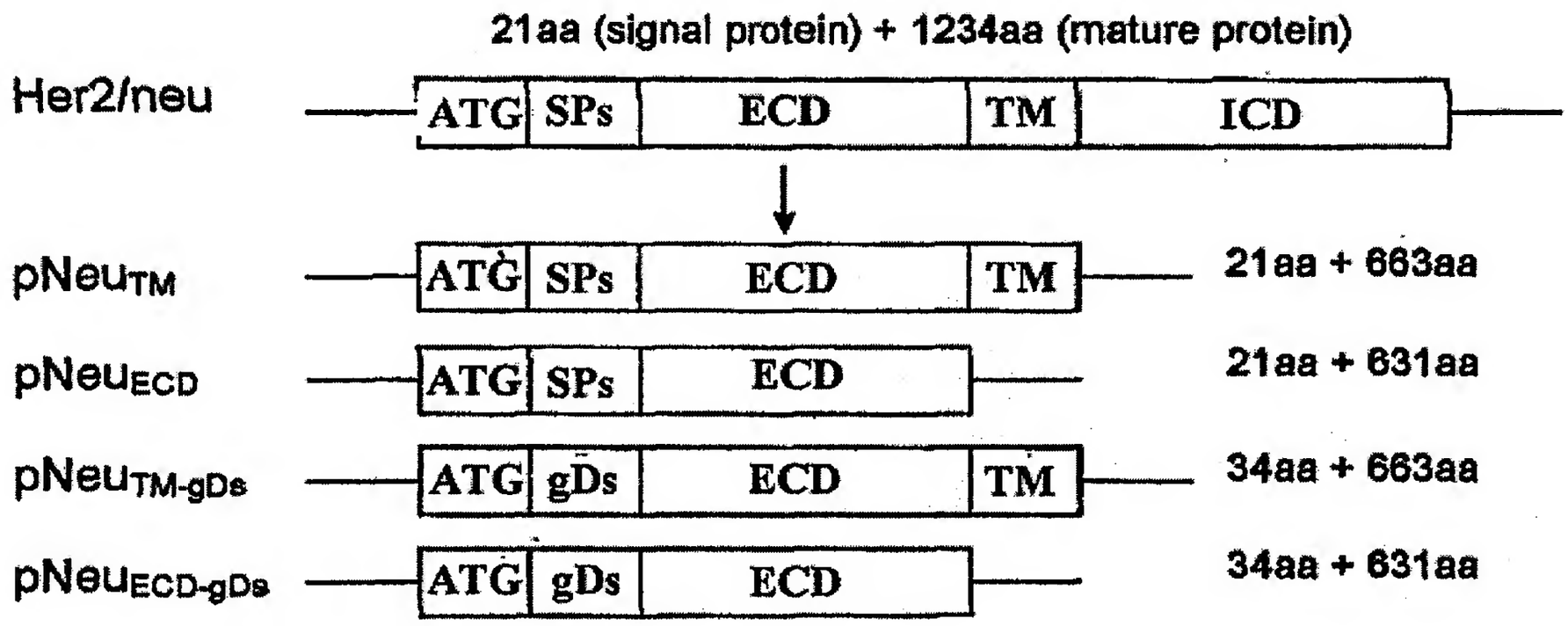


Fig. 1b

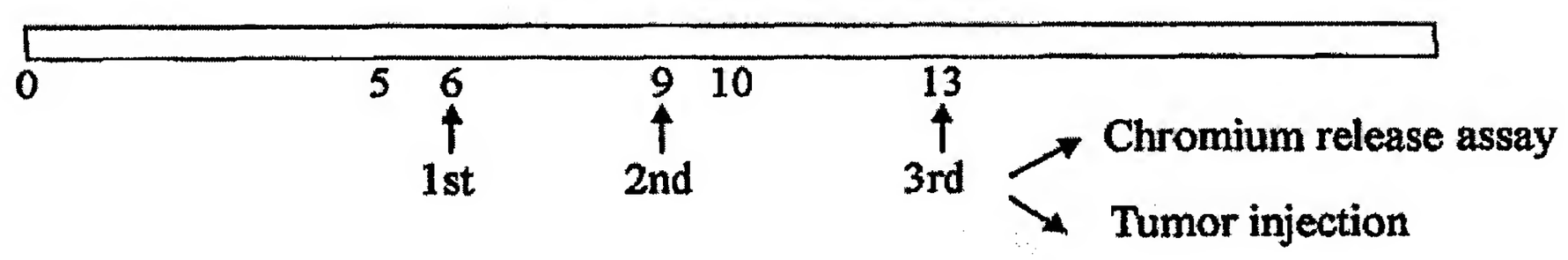


Fig. 2

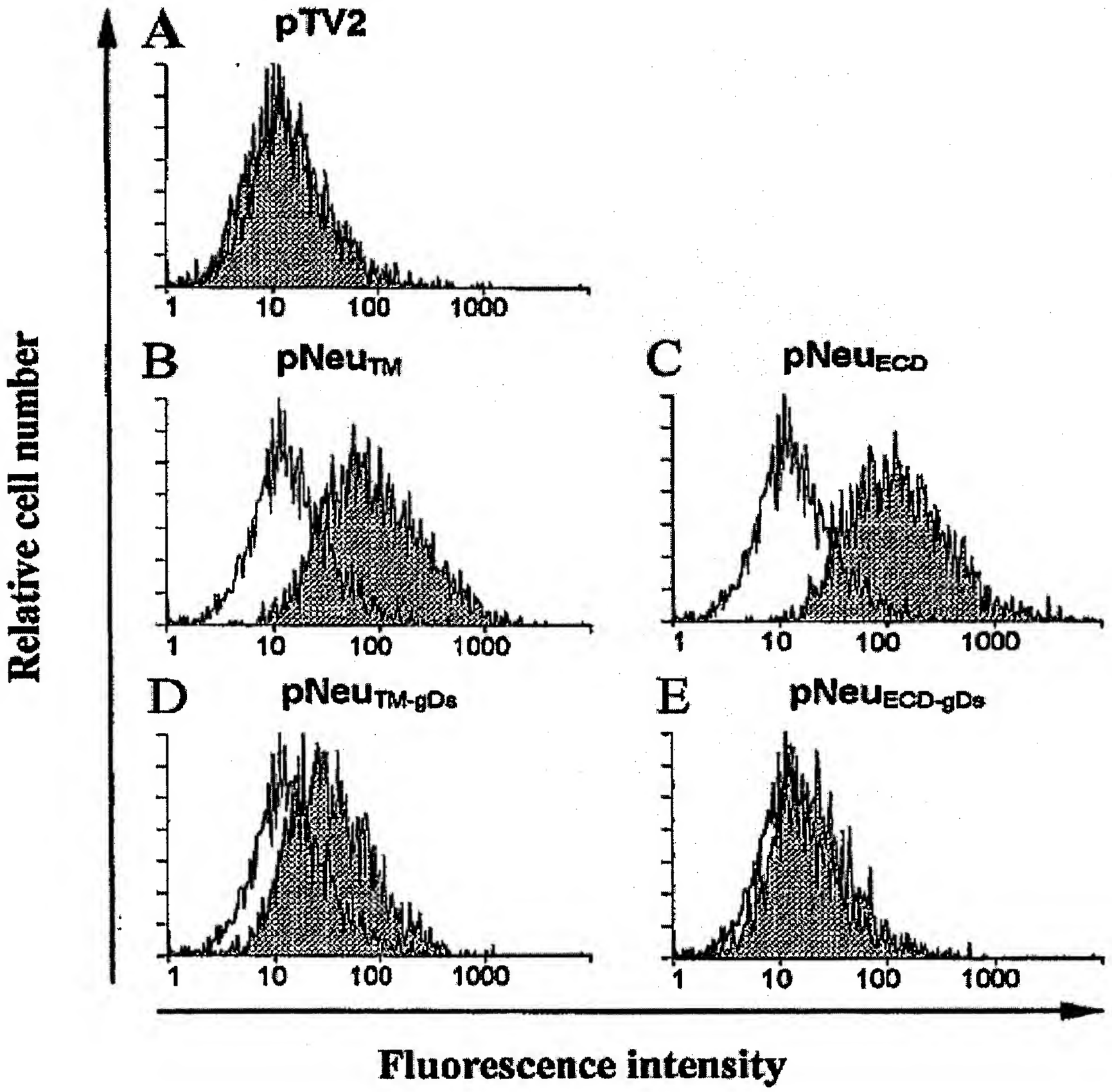

A
pTV2
1
10
100
1000
B
pNeu$_{TM}$
C
pNeu$_{ECD}$
D
pNeu$_{TM-gDs}$
E
pNeu$_{ECD-gDs}$
Relative cell number
Fluorescence intensity

Fig. 3

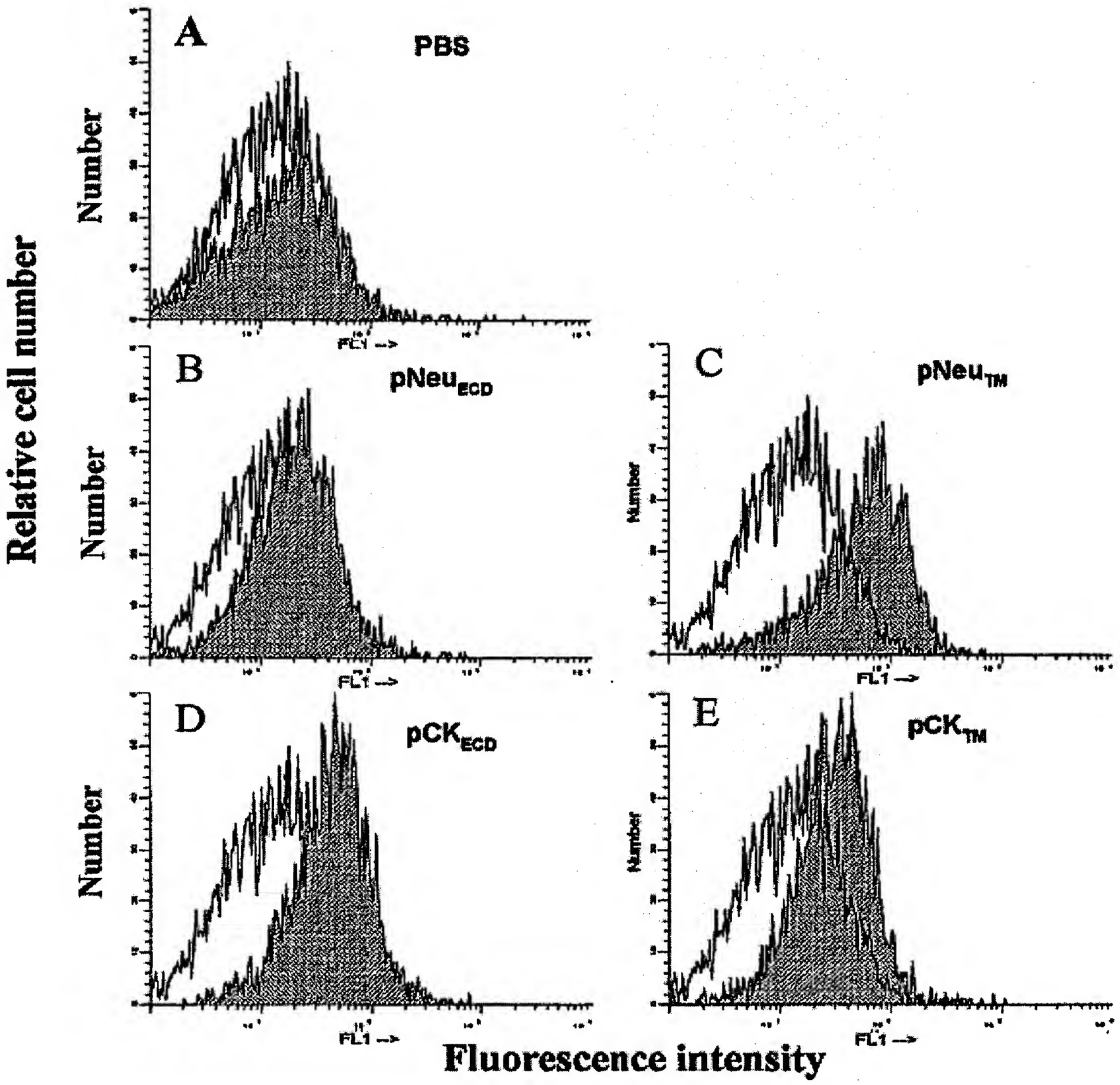

A
PBS
B
pNeu$_{ECD}$
C
pNeu$_{TM}$
D
pCK$_{ECD}$
E
pCK$_{TM}$
Number
Relative cell number
FL1 →
Fluorescence intensity

Fig. 4

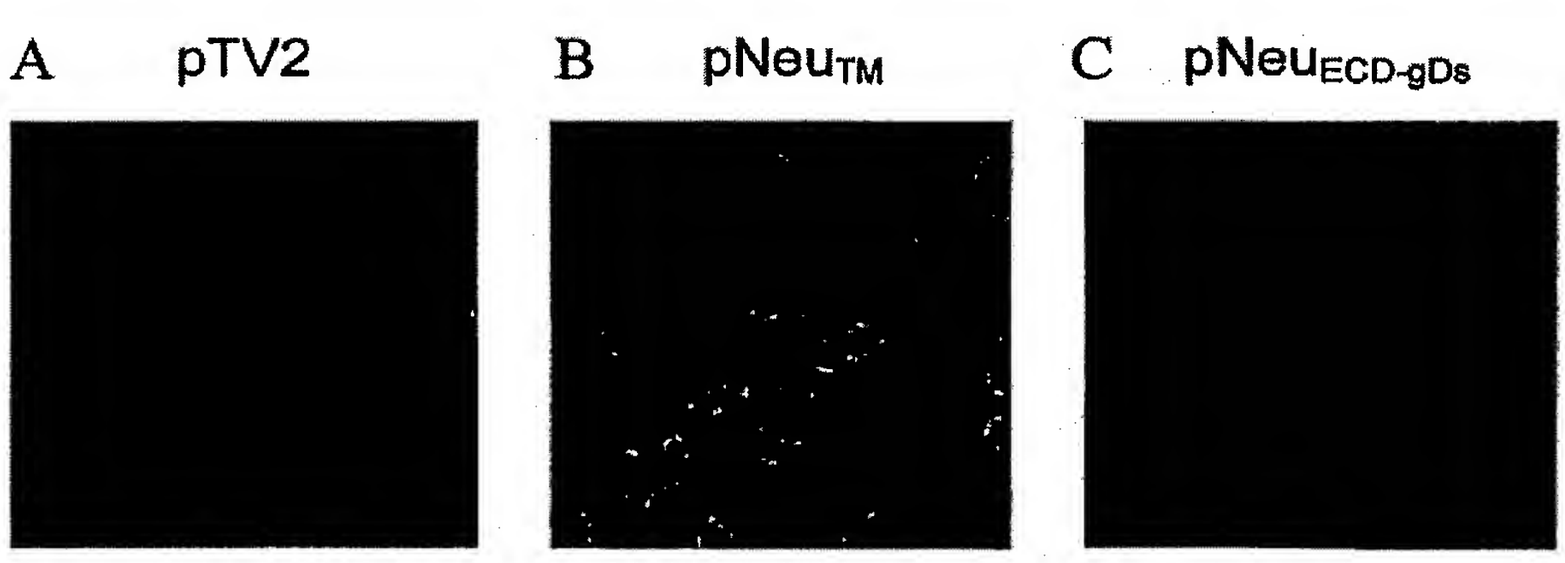

A pTV2
B pNeu$_{TM}$
C pNeu$_{ECD-gDs}$

Fig. 5

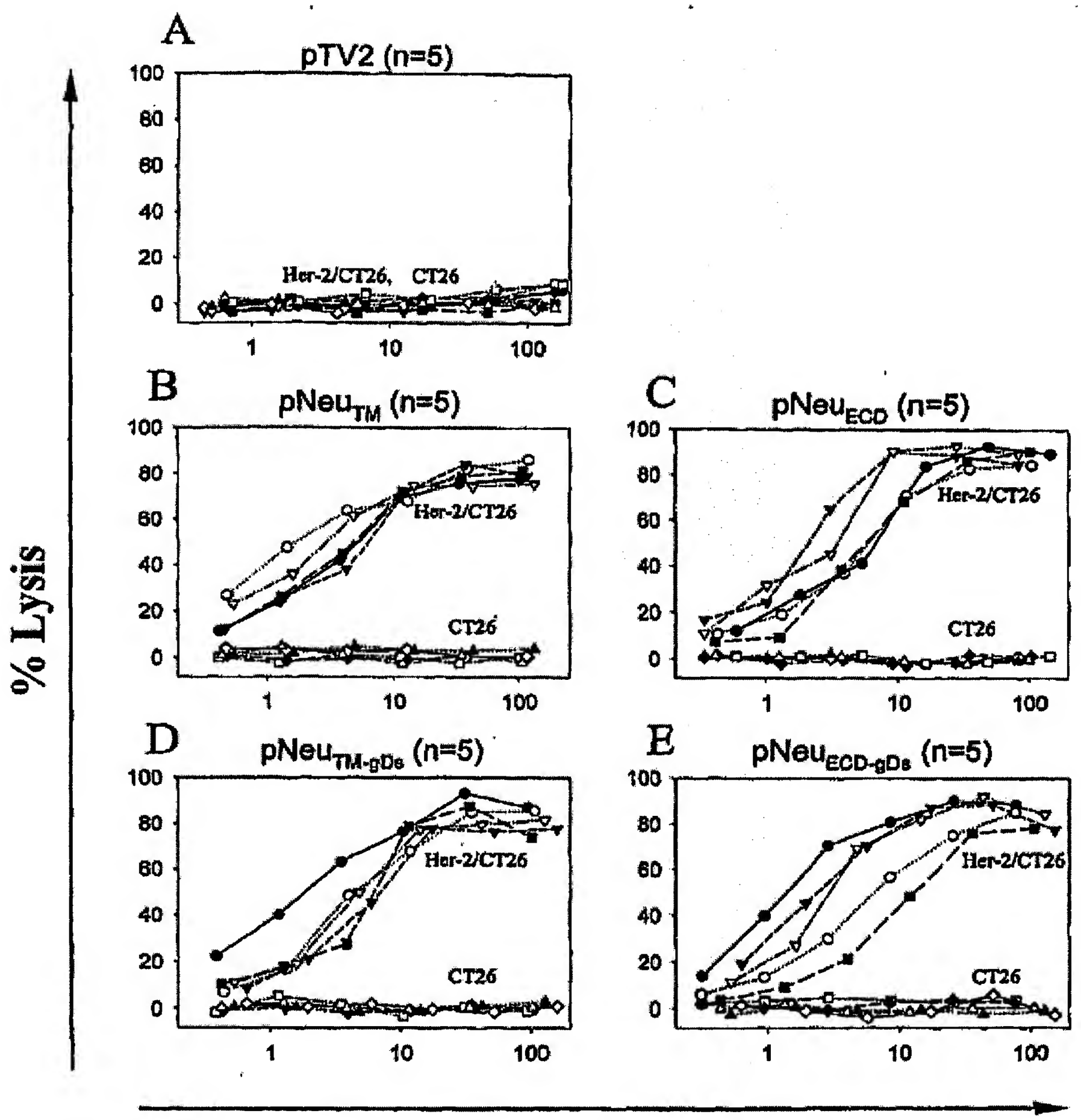

A
pTV2 (n=5)
Her-2/CT26, CT26
B
pNeu$_{TM}$ (n=5)
C
pNeu$_{ECD}$ (n=5)
D
pNeu$_{TM-gDs}$ (n=5)
E
pNeu$_{ECD-gDs}$ (n=5)
Her-2/CT26
CT26
100
80
60
40
20
0
1
10
100
% Lysis
Effector : Target ratio

Fig. 6

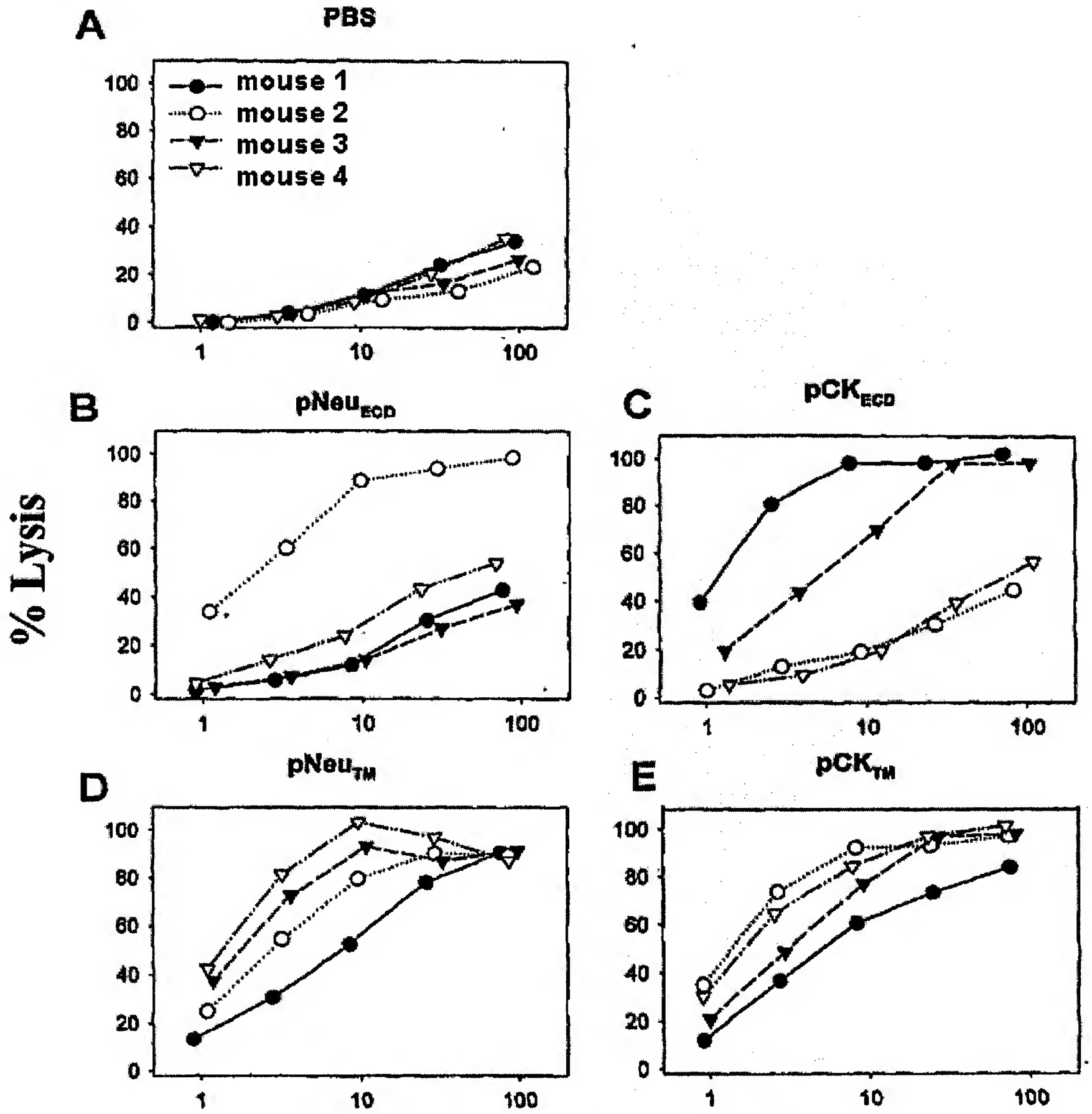

A
PBS
mouse 1
mouse 2
mouse 3
mouse 4
100
80
60
40
20
0
1
10
100
B
pNeu ECD
C
pCK ECD
D
pNeu TM
E
pCK TM
% Lysis
Effector : Target ratio

Fig. 7a

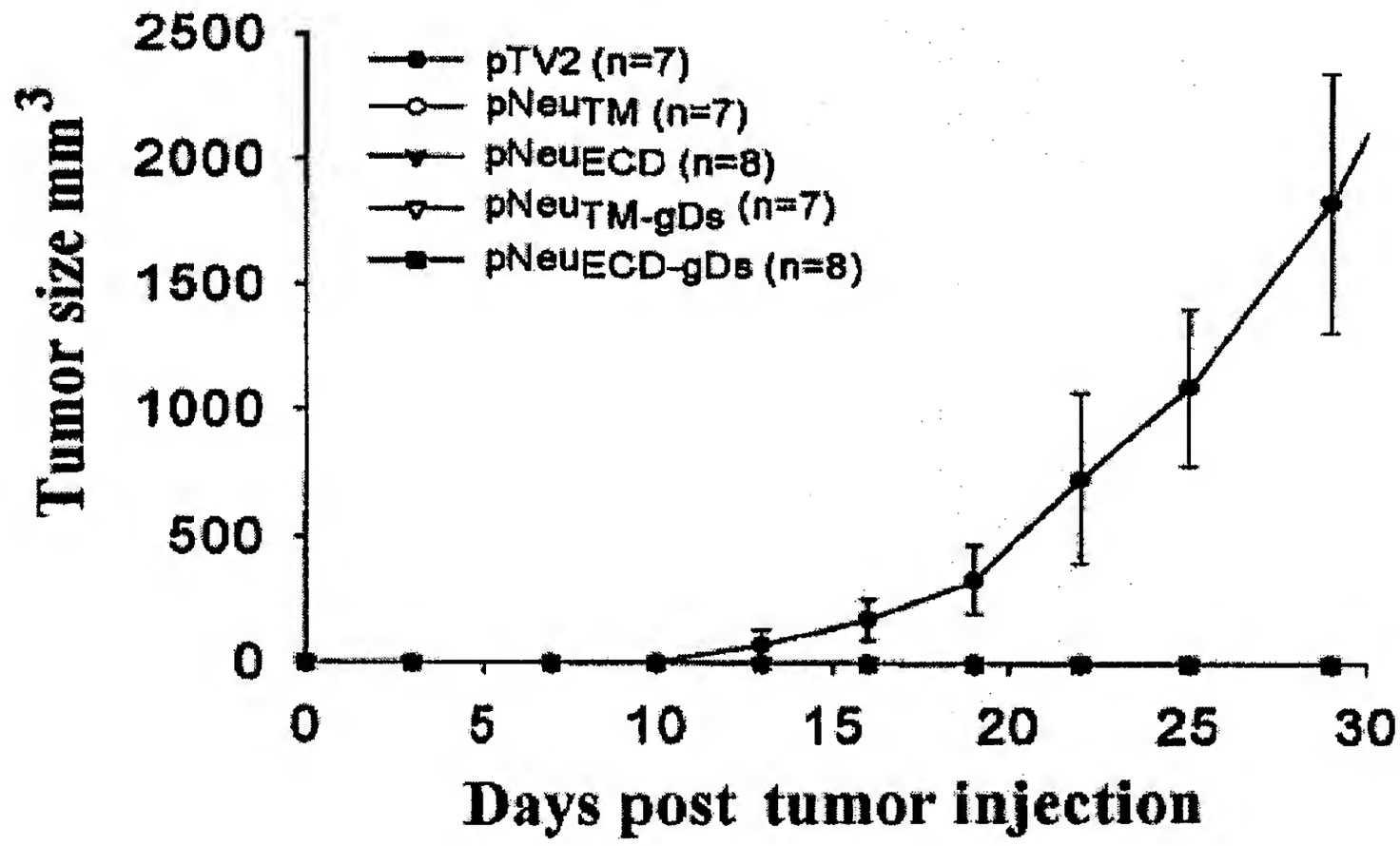

Tumor size mm3
2500
2000
1500
1000
500
0
pTV2 (n=7)
pNeuTM (n=7)
pNeuECD (n=8)
pNeuTM-gDs (n=7)
pNeuECD-gDs (n=8)
0
5
10
15
20
25
30
Days post tumor injection

Fig. 7b

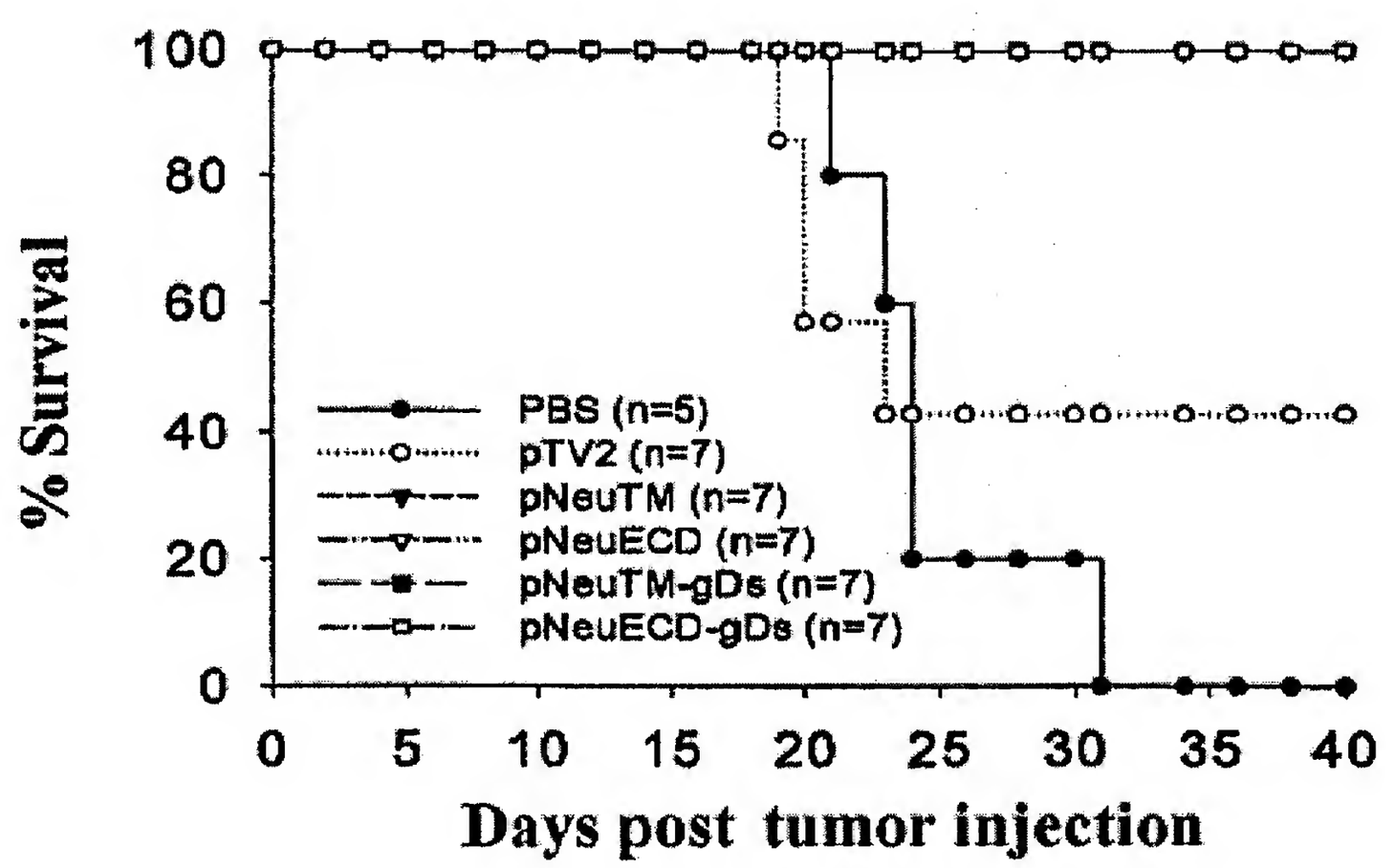

% Survival
100
80
60
40
20
0
PBS (n=5)
pTV2 (n=7)
pNeuTM (n=7)
pNeuECD (n=7)
pNeuTM-gDs (n=7)
pNeuECD-gDs (n=7)
0
5
10
15
20
25
30
35
40
Days post tumor injection

Fig. 8a

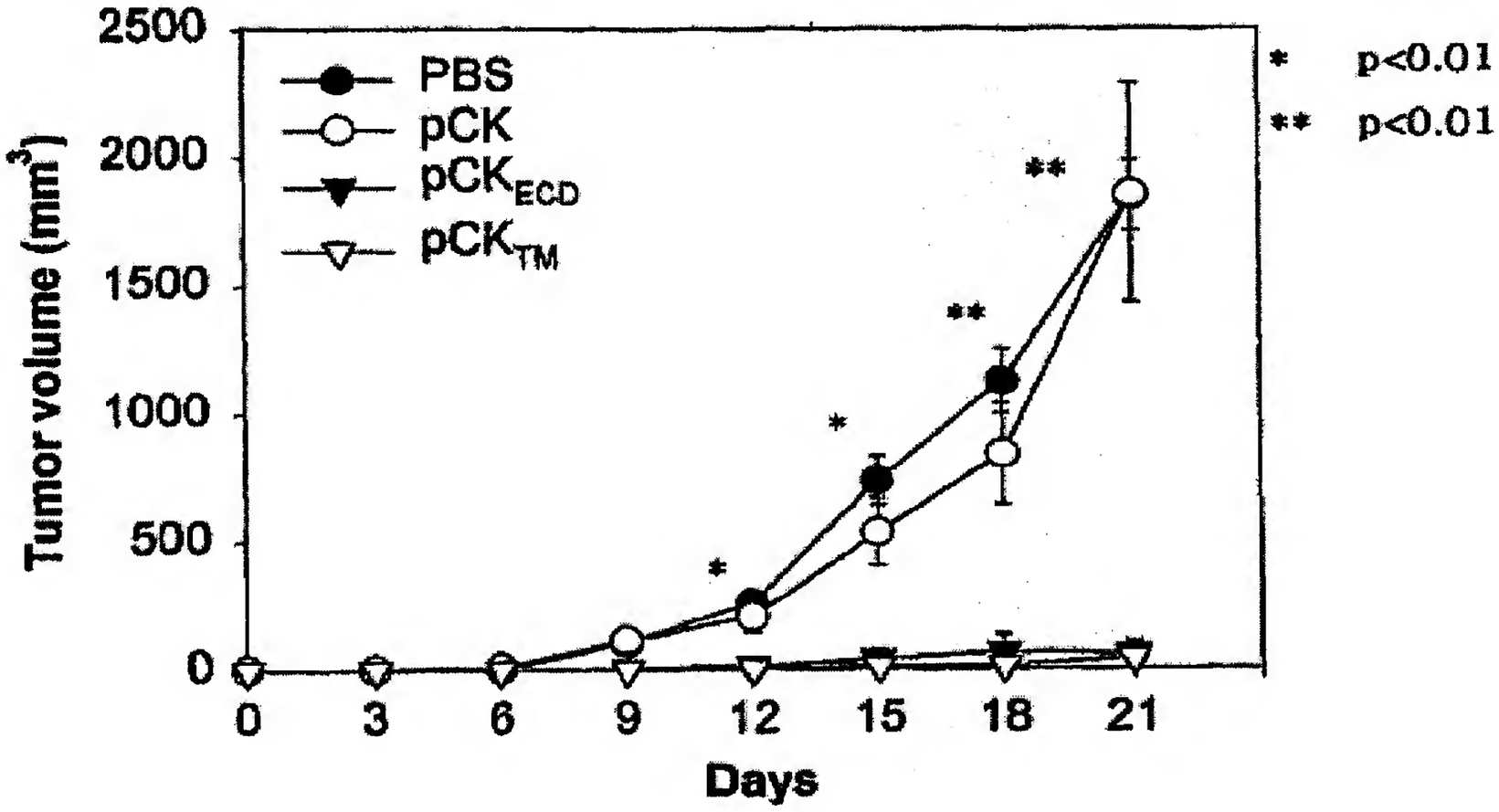

2500
2000
1500
1000
500
0
Tumor volume (mm3)
PBS
pCK
pCKECD
pCKTM
*
**
0
3
6
9
12
15
18
21
Days
* p<0.01
** p<0.01

Fig. 8b

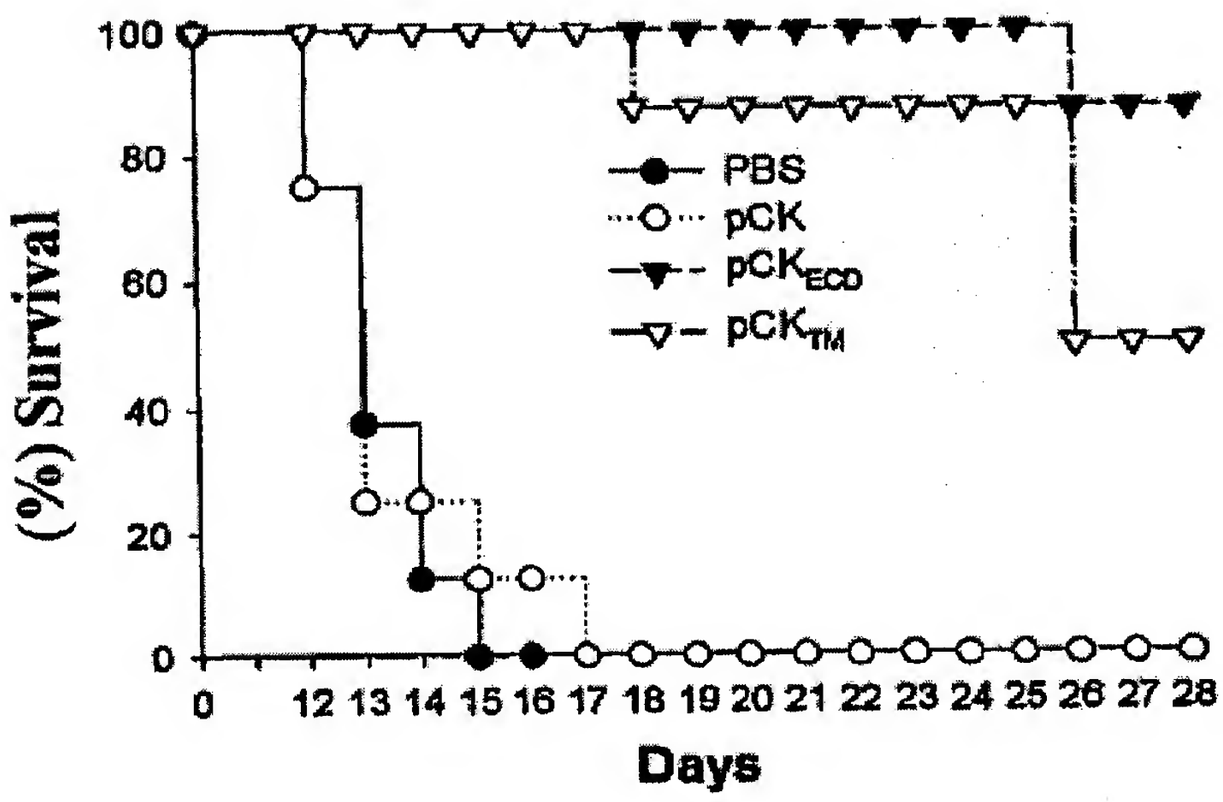

100
80
60
40
20
0
(%) Survival
PBS
pCK
pCKECD
pCKTM
0
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Days

Fig. 9a

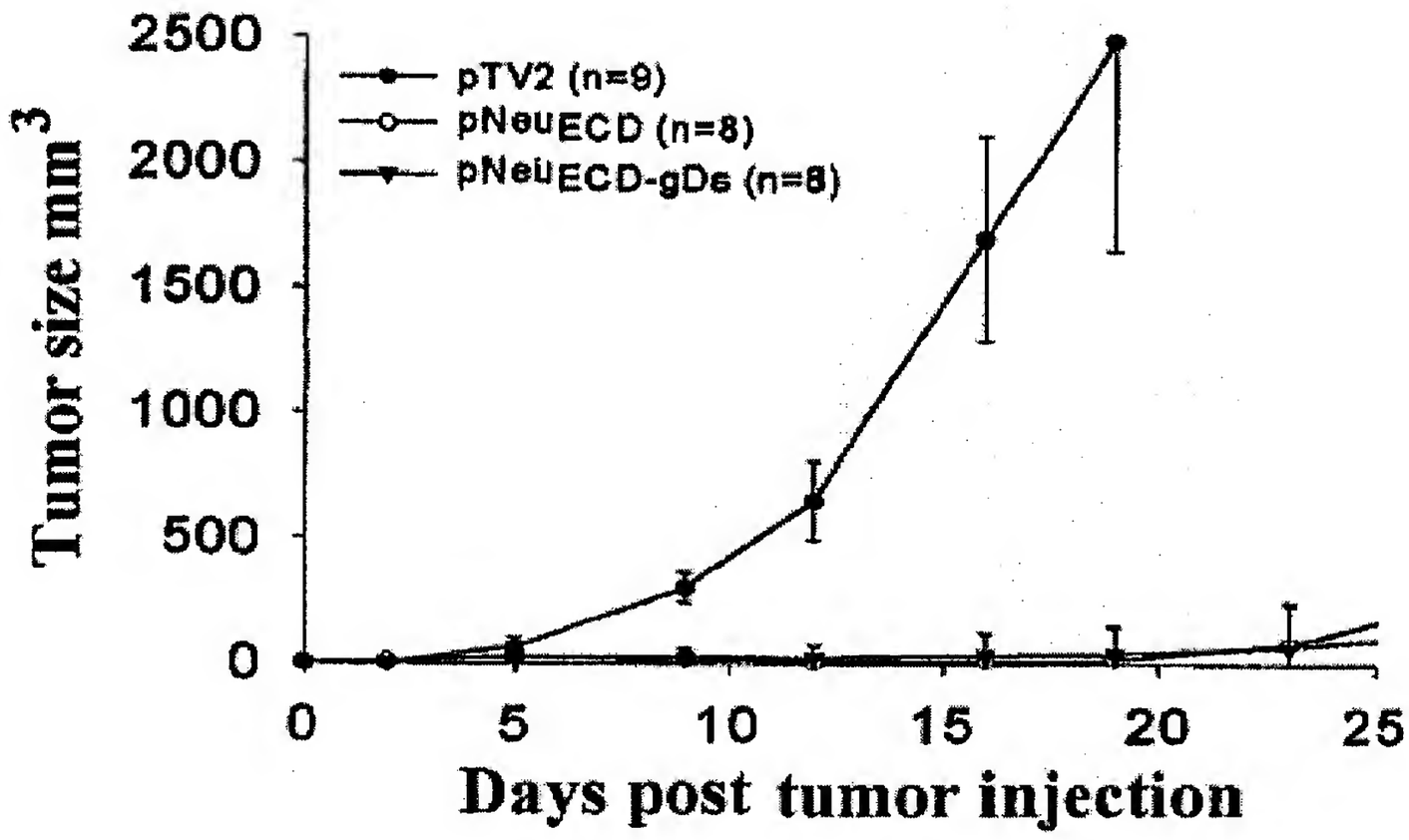

2500
2000
1500
1000
500
0
Tumor size mm3
pTV2 (n=9)
pNeuECD (n=8)
pNeuECD-gDs (n=8)
0
5
10
15
20
25
Days post tumor injection

Fig. 9b

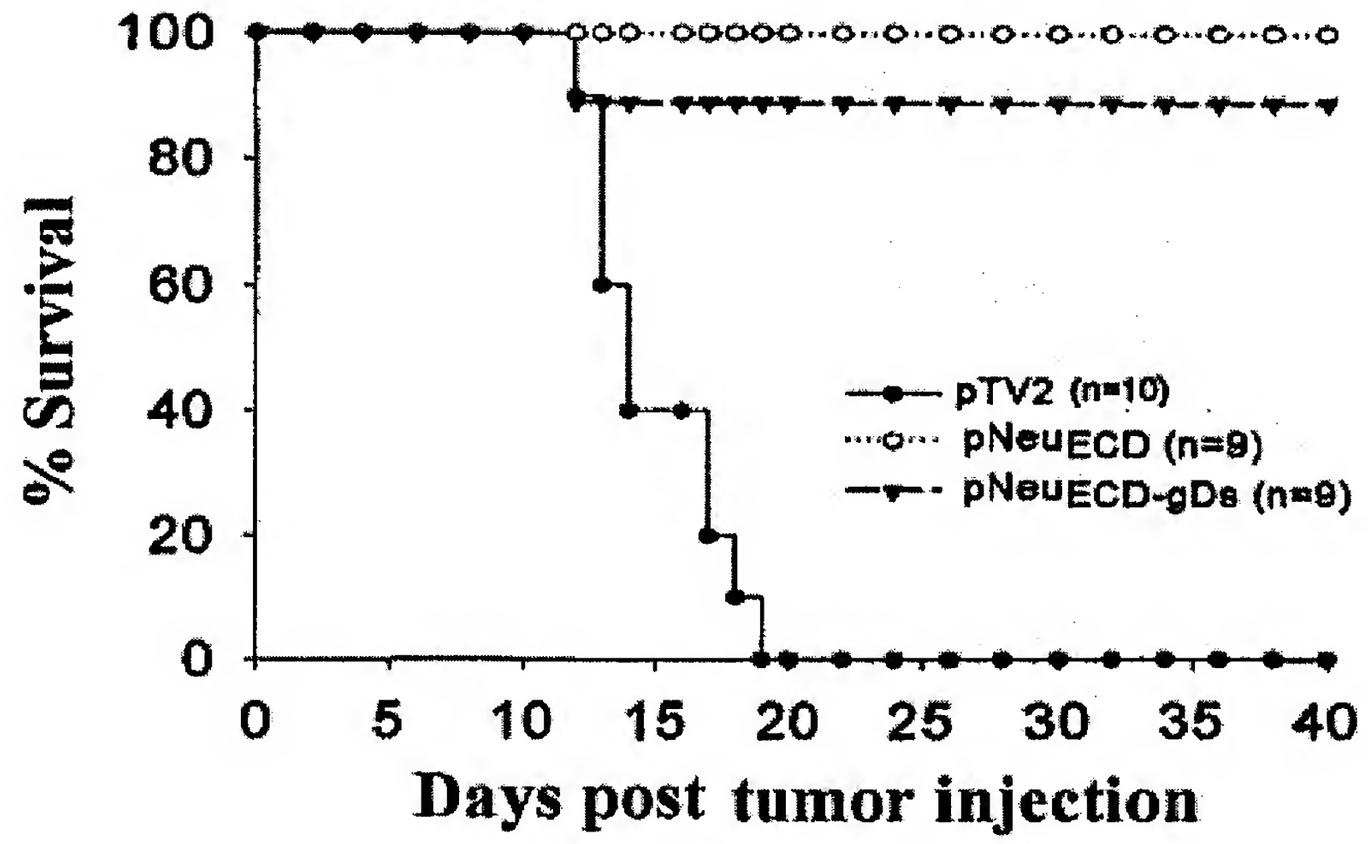

100
80
60
40
20
0
% Survival
pTV2 (n=10)
pNeuECD (n=9)
pNeuECD-gDs (n=9)
0
5
10
15
20
25
30
35
40
Days post tumor injection

Fig. 10a

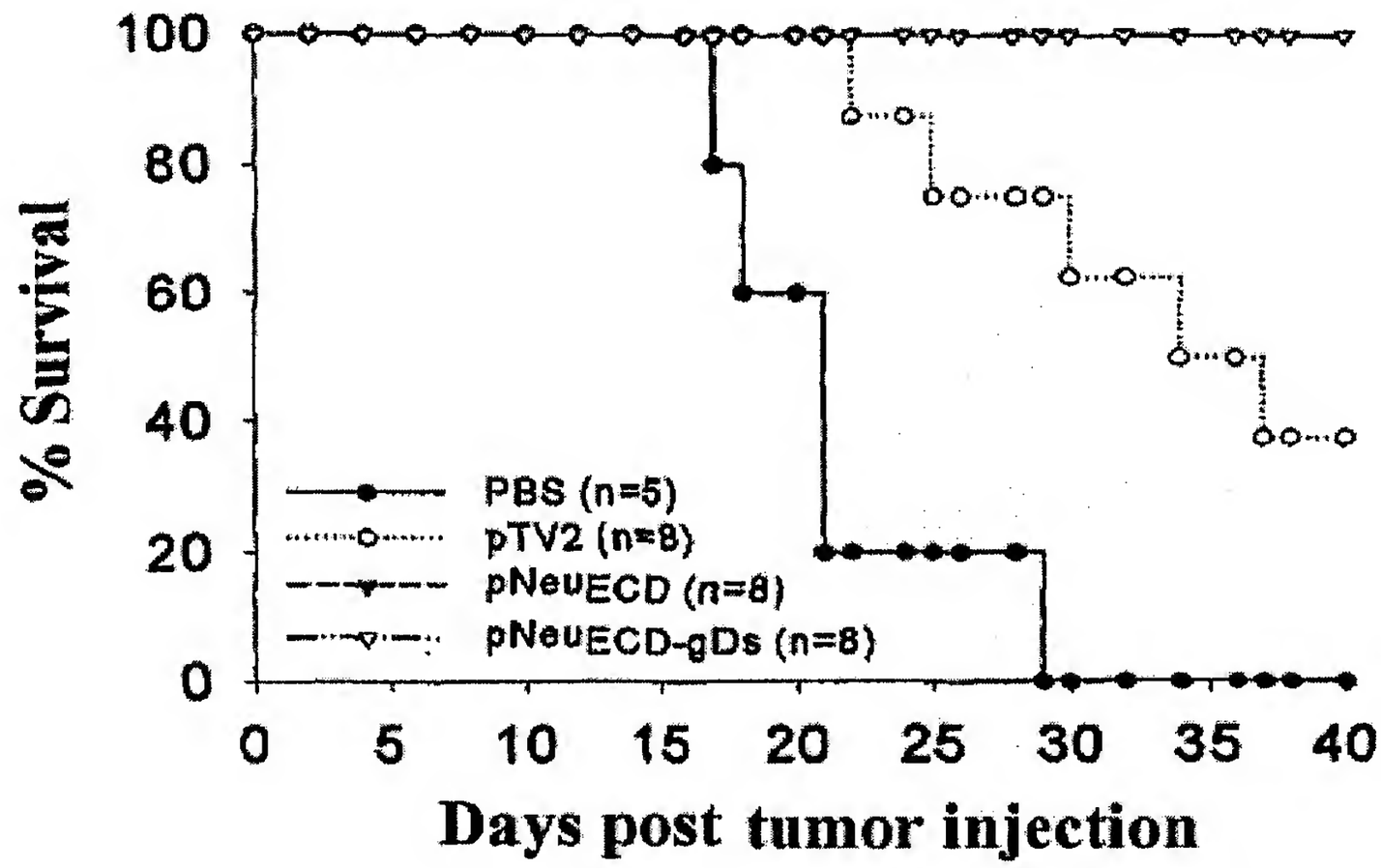


Fig. 10b

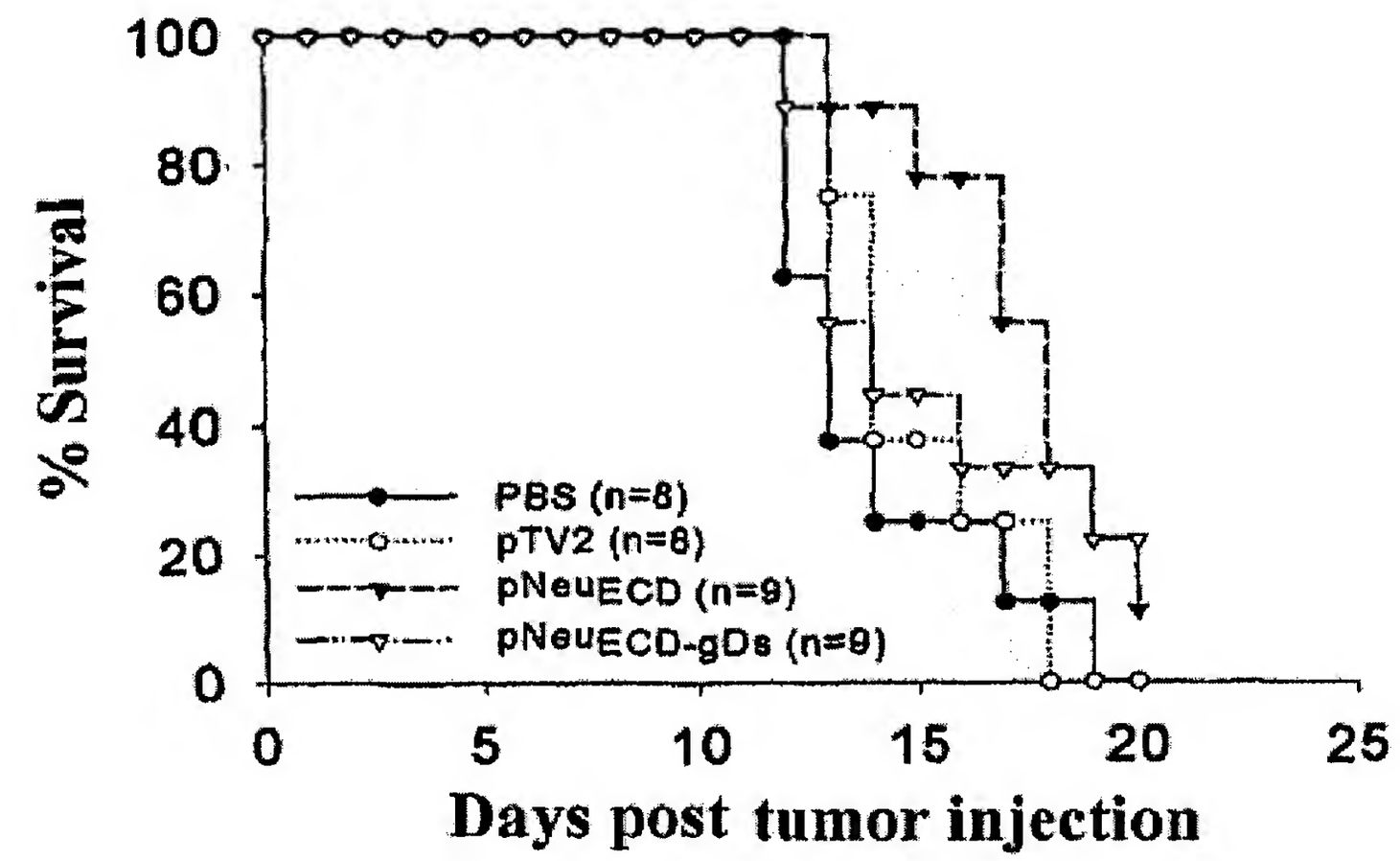
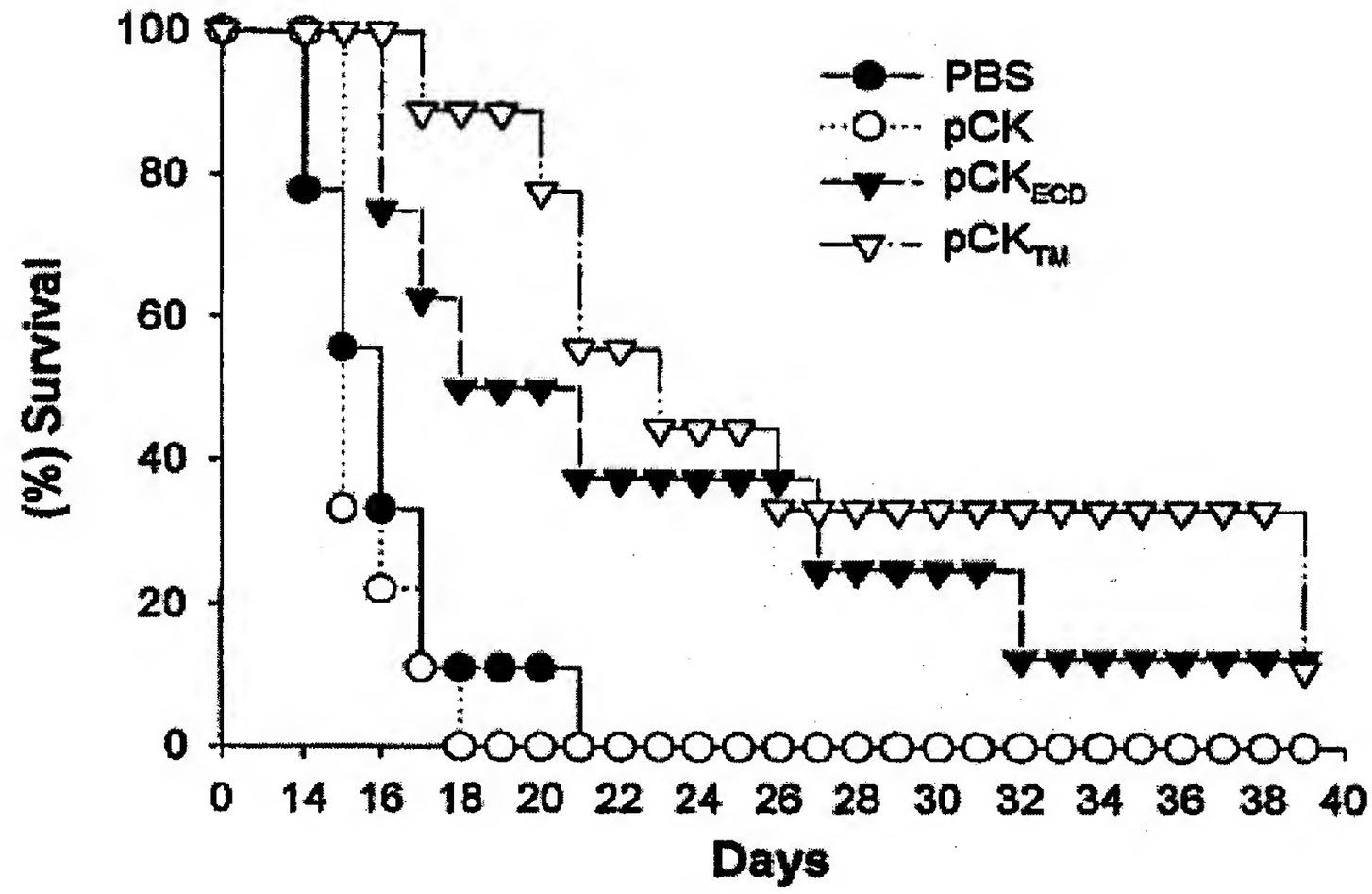


Fig. 11


100
80
60
40
20
0
(%) Survival
PBS
pCK
pCK$_{ECD}$
pCK$_{TM}$
0 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Days

SEQUENCE LISTINGS

<110> PANGENOMICS Co., Ltd

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<160> 9

<170> KopatentIn 1.71

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<212> DNA
<213> human Her-2/neu gene cDNA

<400> 1
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agccatgggg ccggagccgc agtgagcacc atggagctgg cggccttgtg ccgctggggg 180

ctcctcctcg ccctcttgcc ccccggagcc gcgagcaccc aagtgtgcac cggcacagac 240

atgaagctgc ggctccctgc cagtcccgag acccacctgg acatgctccg ccacctctac 300

cagggctgcc aggtggtgca gggaaacctg gaactcacct acctgcccac caatgccagc 360

ctgtccttcc tgcaggatat ccaggaggtg cagggctacg tgctcatcgc tcacaaccaa 420

gtgaggcagg tcccactgca gaggctgcgg attgtgcgag gcacccagct ctttgaggac 480

aactatgccc tggccgtgct agacaatgga gacccgctga acaataccac ccctgtcaca 540

ggggcctccc caggaggcct gcgggagctg cagcttcgaa gcctcacaga gatcttgaaa 600

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gacatcttcc acaagaacaa ccagctggct ctcacactga tagacaccaa ccgctctcgg 720

gcctgccacc cctgttctcc gatgtgtaag ggctcccgct gctggggaga gagttctgag 780

gattgtcaga gcctgacgcg cactgtctgt gccggtggct gtgcccgctg caaggggcca 840

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gactgcctgg cctgcctcca cttcaaccac agtggcatct gtgagctgca ctgcccagcc 960

ctggtcacct acaacacaga cacgtttgag tccatgccca atcccgaggg ccggtataca 1020

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tgtgagaagt gcagcaagcc ctgtgcccga gtgtgctatg gtctgggcat ggagcacttg 1200

cgagaggtga gggcagttac cagtgccaat atccaggagt ttgctggctg caagaagatc 1260

tttgggagcc tggcatttct gccggagagc tttgatgggg acccagcctc caacactgcc 1320

ccgctccagc cagagcagct ccaagtgttt gagactctgg aagagatcac aggttaccta 1380

tacatctcag catggccgga cagcctgcct gacctcagcg tcttccagaa cctgcaagta 1440

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